Vascular Remodeling of the Mouse Yolk Sac Requires Hemodynamic Force

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Abstract

The embryonic heart and vessels are dynamic tissues that form and remodel while functional. Much has been learned about the genetic mechanisms underlying the development of the cardiovascular system, but we are just beginning to understand how changes in heart and vessel structure are influenced by mechanical forces such as those derived from fluid flow. Recently, several mutations in mice have indicated that vessel remodeling in the yolk sac is secondarily effected when cardiac function is reduced or absent. These studies show that proper circulation is required for vessel remodeling, but left open questions about whether the role of circulation is to provide mechanical cues, to deliver oxygen or to circulate signaling molecules. Here we have used time-lapse confocal microscopy to define the role of fluid-derived forces in vessel remodeling in the developing murine yolk sac. We have used these tools to characterize flows in normal embryos and in embryos with impaired contractility ($MLC2a^{-/-}$; Huang et al, 2003). We found abnormal plasma and erythroblast circulation in these embryos which led us to hypothesize that the entry of erythroblasts into circulation is a key event in triggering vessel remodeling. We tested this by sequestering erythroblasts in the blood islands and found that vessel remodeling depends on erythroblast flow. Further, we rescued remodeling defects in these embryos by increasing the viscosity of the blood. These data show that fluid-derived forces that depend on viscosity are necessary and sufficient to induce vessel remodeling in the mammalian yolk sac.

Introduction

The cardiovascular system is the first organ system to develop in vertebrate embryos. In the mouse, mesodermally-derived cardiac progenitor cells coalesce at the midline of the embryo to form a cardiac crescent, then a linear heart tube that begins to beat by early day 8 (Harvey et al., 1999; Ji et al., 2003; Tam and Schoenwolf, 1999). Concurrent with the initial formation of the heart, blood and vessels first form in the extraembryonic yolk sac. Primitive erythroblasts and angioblasts can be detected within the blood islands in the proximal yolk sac by 7.5 dpc. By 8.5 dpc, a capillary plexus consisting of a polygonal network of channels lined by differentiating endothelial cells extends beyond the blood islands, covering much of the yolk sac (Coultas et al., 2005; Drake and Fleming, 2000; Ferkowicz et al., 2003; Fraser et al., 2005; Risau and Flamme, 1995). The first movements of blood cells mark the onset of blood circulation by the 4-5 somite stage (Ji et al., 2003; McGrath et al., 2003), ultimately transporting erythroblasts that differentiate in the yolk sac into the embryo. The subsequent development of the heart and blood vessels occurs in the presence of blood circulation. Over the next 24 hours chamber formation and looping is evident in the heart (Harvey et al., 1999) and the primitive vessels of the yolk sac undergo remodeling to form a branched, hierarchical network of large and small caliber vessels surrounded by smooth muscle cells (see (Armulik et al., 2005) for review).

Many of the genes necessary for the development of a functional cardiovascular system have been identified using large-scale mutagenesis screens or by engineering single gene mutations using model systems such as the mouse (Harvey, 1999; Olson, 2002; Olson and Srivastava, 1996; Solloway and Harvey, 2003) and zebrafish (Stainier, 2001; Trinh le and Stainier, 2004). The large number of genes necessary for heart and vessel development identified in model systems suggests that complex signaling mechanisms are required (see(Argraves and Drake, 2005)). In fact, just one aspect of development, vascular remodeling in the murine yolk sac, depends on over 50 known genes (unpublished observation) including members of the TGF-beta, Notch, VEGF, Hedgehog, retinoic acid signaling pathways (see(Argraves and Drake, 2005)). A clear understanding of the role of individual genes can often be hampered by the fact that both primary and secondary abnormalities may result from a single gene mutation. For

instance, abnormal vascular remodeling can result from mutations in genes that normally function within endothelial cells or can be caused by mutations in genes needed solely for proper cardiac contraction. Null mutations in several genes required for cardiac function, including Ncx1 (Koushik et al., 2001; Wakimoto et al., 2000), MLC2a (Huang et al., 2003), Nkx2.5 (Tanaka et al., 1999) and Titin (May et al., 2004) all result in a failure in yolk sac remodeling despite a lack of expression in endothelial cells. Furthermore, vascular abnormalities caused by the deletion of N-cadherin can be rescued by cardiac specific expression of either N- or E-cadherin (Luo et al., 2001). To explain these effects, it has been proposed that normal blood flow is necessary for vascular remodeling. Numerous studies from avian embryos, dating back to well over a century ago, support this hypothesis since the surgical manipulation blood flow can lead to a wide range of abnormalities in heart and vessel development such as hypoplastic left heart syndrome, transposition of the great arteries, atrial and septal defects and pharyngeal arch abnormalities (Clark and Hu, 1982; Clark et al., 1984; Clark, 1918; Congdon and Wang, 1926; Gessner, 1966; Gessner and Van Mierop, 1970; Harh et al., 1973; Murray, 1926; Rychter, 1962; Thoma, 1893).

While many studies show that blood flow is required for vessel and cardiac remodeling, what continues to be debated is the reason why blood flow is important (Conway et al., 2003; Coultas et al., 2005; Huang et al., 2003; May et al., 2004). For instance, blood flow could be required because it delivers nutrient or oxygen support to tissues that are necessary for remodeling. Hypoxic conditions could damage endothelial cells directly or alter the levels of necessary growth factors such as VEGF which could impair remodeling (Conway et al., 2003; May et al., 2004). However, early mouse embryos (8.5dpc), like early chick, frog and zebrafish embryos, can be cultured in the presence of carbon monoxide, which competes for oxygen binding to hemoglobin, without effecting the initial stages of vasculogenesis or vascular remodeling (Jones, Fraser and Dickinson, unpublished) (Cirotto and Arangi, 1989; Pelster and Burggren, 1996; Territo and Burggren, 1998), raises questions about the requirement for blood-derived oxygen. Even though oxygen delivery may not appear to be critical for some events at early stages, it could be argued that early blood flow transports soluble nutrients, such as growth factors and cell signaling molecules that are required for

vascular remodeling (Conway et al 2003). This would mean that circulation would be necessary to distribute such a factor, perhaps from a source in the embryo proper to more peripheral tissue. A third hypothesis is that circulation imparts force on plexus endothelial cells and that this mechanical force is necessary to activate the cell-signaling cascades necessary for vessel remodeling and continued embryonic development. Blood flow exerts two types of force on vessels: shear stress, the frictional force tangential to endothelial cells, and circumferential strain, a force perpendicular to the direction of flow and related to changes in intravascular pressure. Numerous studies have shown that cultured endothelial cells can perceive these forces and can respond by changing morphology, activating intracellular kinases, and inducing gene expression (for review, see (Kamiya and Togawa, 1980; Lehoux and Tedgui, 1998; Li et al., 2005; Orr et al., 2006; Resnick et al., 2002). Further support for this hypothesis comes from the fact that several factors known to be influenced by shear stress are essential for normal cardiovascular development, such as PDGF-β (Resnick et al., 1993), connexin43 (Gabriels and Paul, 1998), and Flk1 (Gabriels and Paul, 1998). Also, shear stress levels in the mouse yolk sac are within the range known to induce gene expression and morphological changes in cultured endothelial cells (Jones et al., 2004). Despite the circumstantial evidence for flow-derived force in vascular remodeling, no direct evidence exists to show that mechanical signals are sufficient to drive remodeling or to exclude the possibility that the local oxygen supply or soluble, paracrine factors provided through circulation act as a trigger.

To better understand the role that circulation plays during early embryonic mouse development we have used whole embryo culture and time-lapse confocal imaging (Jones et al., 2002) to visualize and quantify blood flow in early embryos. We first used these methods to analyze the dynamic events at the onset of circulation in normal mouse embryos. Next, we characterized aberrant flows in Myosin Light Chain 2a (MLC2a) null embryos to understand how secondary remodeling defects are generated by impaired contractility. Finally, we tested whether altering the mechanical properties of the early blood can phenocopy the vascular remodeling defect seen in MLC2a embryo. These studies have revealed that there are two temporal phases of flow during early embryogenesis. Slow, but steadily increasing plasma flow is observed, beginning with

the first heart beats. Erythroblast circulation begins several hours after the heart begins to beat and starts with the intermittent flow of individual blood cells. Using the same methods, we analyzed flow events in MLC2a mutant embryos and found that both phases of blood flow are effected. Early plasma flow is greatly reduced and erythroblasts move in an oscillatory, rather than laminar flow pattern, with reduced velocity. These results lead us to hypothesize that the laminar flow of erythroblasts is necessary for remodeling. To test this, we experimentally manipulated cells within the blood islands in early somite embryos to prevent the entry of erythroblasts into the circulation, removing the primary viscous component of the blood. Both vascular remodeling and embryo turning were impaired when erythroblasts were prevented from circulating, showing that the flow of erythroblasts is necessary for remodeling. Normal remodeling and turning behaviors were rescued in treated embryos when the viscosity of the plasma was increased, showing that mechanical forces are sufficient to induce remodeling. Thus, we have used novel methods to analyze and manipulate blood flow in early mouse embryos to answer a century-old question about the role of mechanical force in vessel remodeling in vivo.

Materials and Methods

Embryo preparation and Time-lapse Microscopy

Breeding pairs of MLC2a$^{+/-}$;Tg(ε-globin::GFP) mice (Dyer et al., 2001; Huang et al., 2003) were mated together and the presence of a vaginal plug in the morning was taken as 0.5 dpc. Embryos were collected on the morning of the eighth day and cultured for time-lapse microscopy as previously described (Jones et al., 2002).

To investigate changes in erythrocyte flow, single images were taken every 6 minutes at a magnification of 20x (Plan-Neofluar 20x/0.5NA) on a Zeiss LSM 5 PASCAL. Real time images of oscillatory motion were taken with 40x magnification, and individual cells were tracked manually by connecting the center of the same cell in subsequent images. Overlay of tracking images was performed using Adobe Photoshop.

Measurements of plasma flow

Embryos were removed from the dam at early 8.5 dpc. They were allowed to recover in dissecting media (90% DMEM/F12, 9% fetal bovine serum, 1% pen/strep) for 30 minutes at 37°C (Jones et al., 2002). A pulled quartz needle (Sutter Instruments) was filled with 10,000 MW Texas Red fluorescent dextran (Molecular Probes, No. D-1828) and a picospritzer II (General Valve Corp.) was used to inject nanoliter volumes of dye into the heart tube. Embryos were allowed to recover for up to ten minutes and imaged with the Zeiss LSM PASCAL 20x magnification to determination of the onset of plasma flow.

Fluorescence Recovery After Photobleaching

After dissection, embryos were allowed to recover in dissecting media for 30 minutes at 37°C. A pulled quartz needle was filled with 50mg/ml, 10,000 MW fluorescein dextran (Molecular Probes, No. D-1821) and nanoliter volumes of dye was injected into the heart tube. Embryos were transferred to Nunc Lab-Tek chambers (No. 155380) with culture media (49% DMEM, 49% DMEM, 1% 1M HEPES, 1% pen/strep) (Jones et al., 2002). The embryos were allowed to recover in a tissue incubator at 37C for up to one hour to ensure that dye was present in all vessels and that heart rates were normal. The microscope (Zeiss LSM5 PASCAL or Zeiss LSM 510 META) was preheated to 37°C using a heater box (Jones et al., 2002). With a 20x lens, an initial image of the plexus was taken, and the location of the bleach recorded. Scan speeds were set to 100 msec per frame and the aperture was set fully open. The region of interest was scanned several times to obtain initial fluorescence levels. The laser power was increased to 100% to bleach and subsequently returned to the original setting for measurements. 500-600 frames were collected for each data set. Embryos were transferred to separate wells and somites were counted.

Mean fluorescence with respect to time for the region of interest was tabulated by the LSM software and exported to a spreadsheet. The recovery curve was fit to the equation [Soumpasis, 1983]:

$$\frac{F(t)-F_0}{F_F-F_0} = e^{\frac{-2\tau}{t}}\left[I_0\left(\frac{2\tau}{t}\right)+I_1\left(\frac{2\tau}{t}\right)\right]$$

Where F(t) is the fluorescence intensity, F_0 is the initial post-bleach fluorescence intensity, F_F is the final level of fluorescence recovery, I_0 and I_1 are zeroth and first order Bessel's Functions, and τ is the characteristic diffusion time. The bleach area was divided by characteristic diffusion time to give the measured diffusion. The baseline for pure diffusion was obtained by injecting embryos with fluorescein-dextran as described, stopping the heart by chilling the embryos at 4°C for 1 hour, re-heating the embryos to 37°C and performing FRAP. Absence of heart beat was verified prior to measuring FRAP.

Erythroblast Immobilization

Embryos were dissected at approximately the 5 somite stage. A 30% acrylamide/bis-acrylamide (#161-0158, BioRad) was diluted 1:1 with 2X phosphate buffered saline (PBS) and 1.2 v/v% ammonium persulfate (stock 20 w/v%) solution was prepared. Blood islands were visualized using a Tg(ε-globin-GFP) transgenic line (Dyer et al., 2001) on a fluorescent dissecting scope. The solution was microinjected directly into the blood islands by applying a small positive pressure to the injection needle while gently rolling the embryo with the tip of the needle. The media was exchanged and the embryos were allowed to recover for approximately 15 minutes. The polymerizing agent TEMED (#161-0801, BioRad) was diluted 1:1 with 2X PBS. The optimal dilutions for both TEMED and acrylamide were determined via dilution series (data not shown), and the combinations producing an instantaneous soft gelatinous matrix with the lowest chemical concentration was chosen. The polymerizing solution was loaded into the microinjector pipette and a small positive pressure was applied to create a small fluid stream of at the pipette tip. The tip was then gently touched to the surface of the blood islands to allow polymerization of the acrylamide solution. The media was subsequently changed and the embryos were allowed to recover in a standard tissue culture incubator (37°C, 5% CO_2) for 30 minutes before placing them in roller culture in a rat serum rich media (Jones et al 2002) as previously described in (Tam, 1998). Three sets of controls were performed: a) uninjected embryos, b) embryos injected only with the acrylamide solution, and c) embryos injected with only TEMED into the blood islands.

To assess embryo development, yolk sac growth and remodeling, embryos were imaged at the same magnification with a Zeiss Axiocam mounted on a Zeiss Lumar stereomicroscope equipped for fluorescence imaging. 10,000 MW Texas Red fluorescent dextran (Molecular Probes, No. D-1828) injected into the yolk sac vessels (see above) was used to produce a fluorescent angiogram to better visualize remodeling. The image scale was calibrated to an

etched glass standard and the yolk sac perimeter was traced using Zeiss Axiovision software to determine the area. Turning was scored on a scale of 1 to 5 (1=not turned, 5=fully turned) and yolk sac vessel remodeling was scored on a scale of 1 to 5 (1=regular polygonal structure throughout yolk sac, 5=hierarchical branching pattern of arteries and veins present throughout yolk sac). Embryos were randomly assigned a number so that the experimental group was not identifiable at the time of scoring and the embryos were matched to their experimental groups until after scoring.

Hetastarch Injection

Hetastarch (Sigma Aldrich, no. H2648) consists of a high molecular weight hydroxyethyl starch in a physiologic saline solution (6% in 0.9% NaCl). Approximately 1-2 hours of recovery after TEMED administration, embryos received an intracardiac picoliter injection of Hetastarch, the media was changed, and embryos were returned to the incubator.

Results

Initiation of Blood Flow in Wild-Type Embryos

Previous studies have shown that erythroblasts enter circulation in a stepwise pattern (McGrath et al., 2003). These and other studies (Ji et al., 2003) have examined the location of erythroblasts in fixed embryos at different stages to characterize the onset of mammalian circulation. With the advent of methods for time-lapse analysis of embryos at these early stages (Jones et al., 2002) and the availability of the Tg(ε-globin-eGFP) transgenic line in which GFP is expressed within primitive erythroblasts (Dyer et al., 2001), circulation within the early mouse embryo can be directly visualized using high spatial resolution methods. To observe the onset of circulation in mouse embryos, Tg(ε-globin-eGFP) embryos (n=3) were placed in culture at the 5-6 somite stage (Movie 1, Figure 1). During the first hour, the heart beat is evident but erythroblasts remain confined to the blood islands and are not observed in the circulation (Figure 1A, H). Occasionally, GFP+ erythroblasts are observed outside the blood islands, often as single, stationary cells that are adherent to the walls of the lumen of the capillary plexus (Figure 1A, red arrow). It is not clear if these cells differentiated at these sites or if these

cells were previously transported via plasma flow and have come to rest. As erythroblasts begin to enter the circulation, the hematocrit, or volume percentage of red blood cells in vessels, is initially low and fluctuates from one frame to another (Figure 1B). Throughout the initiation of circulation, many blood cells flow with a net forward motion (Movie 1). Although flow is apparent within most of the vessels, some individual erythroblasts become stationary and group together. In this movie, some groups of erythroblasts remain adherent to one another for up to 3 hours (30 frames, Figure 1, arrows) before dissociating and rejoining circulation. Thus, the early circulation of erythroblasts is stochastic and intermittent and some cells do not consistently remain suspended in the flow.

The onset of circulation is usually defined by the onset of erythroblast circulation. We were interested in determining if significant plasma flow existed prior to the entry of erythroblasts into the capillary network vessels. In agreement with previous findings, we detected a beating heart in embryos as early as the 3 somite stage (Navaratnam et al., 1986). To test whether enough work was performed by the heart to cause plasma to flow from the embryo to the yolk sac, we injected a small volume of fluorescent dextran into the hearts of early somite-stage embryos and examined whether the dextran could be observed in the yolk sac plexus after a 10 minute incubation period at 37C. In 0 and 1 somite stage embryos, injected dextran remained confined to the site of injection in the heart (n= 4, data not shown). Similarly, dextran was not distributed through the yolk sac in most embryos at the 2 somite stage (5 of 6 embryos, Figure 2A-B, D-E). However, dextran was consistently found throughout the capillary plexus in 3-somite embryos (20 of 20 embryos; Figure 2C,F). These results show that a continuous network between the capillary plexus and the embryo is established at least by the time the heart begins to beat and the appearance of dextran in the plexus after such a short incubation time suggests that there is significant plasma flow between the embryo and yolk sac.

To show that the movement of dextran between the embryo and the yolk was the result of circulation and not diffusion, we measured plasma flow in embryos prior to the entry

of erythroblasts using Fluorescence Recovery After Photobleaching (FRAP) (for review, see (Lippincott-Schwartz and Patterson, 2003)). Fluorescent Dextran (see materials and methods) was injected into the heart tube of 3-8 somite stage embryos. After uniform distribution of fluorescent dextran in the yolk sac plexus, a region of interest was bleached and the recovery of fluorescence into the bleached area was measured to calculate the rate of plasma convection. Initially, we used the FRAP technique to measure the rate of diffusion of dextran in plasma using embryos with an arrested heart beat (see materials and methods). The diffusion rate was found to be 51 $\mu m^2/s \pm 7.6$, n=8 at 37° C. In the presence of the heart beat, even at early stages, we observed faster fluorescence recovery of the bleached area and we have termed this measurement the perfusion coefficient (Figure 3). At the 3-4 somite stage, the measured perfusion coefficients ranged between 100 and 500 $\mu m^2/s$ indicating the presence of slow, but significant flow (Figure 3). The variability of plasma flow measurements likely relates to the proximity of the measurement site to the heart, as we would expect plasma flow to be slower further from the heart and faster where there is a shorter, less tortuous path to the heart. Higher perfusion values were measured in later stage embryos (5-6 somite stage), and in these embryos, plasma flow in some yolk sac regions was too fast to measure by FRAP (indicated by asterisk with arrow). Since the circulation system appears to be intact and plasma flow rates increase as embryos mature, it is possible that whole blood flow does not begin until blood cells can remain suspended by the strength of the flow. Also, these experiments indicate that yolk sac endothelial cells are exposed to two different flow regimes in early development, first to plasma flow which increases in velocity steadily during early somite stages, followed by the circulation of erythroblast-containing blood beginning at 5-7 somites.

Erythroblast Circulation in MLC2a -/- embryos

Null myosin light chain 2a mice exhibit defects in atrial contraction and ventricle filling as well as in vascular remodeling (Huang et al., 2003), making this mutant an excellent model to study the relationship between circulation and remodeling. To observe circulation directly, MLC2a mutant mice were crossed with Tg(ε-globin-eGFP) mice

(Dyer et al., 2001). Confirming previous findings, fluorescence images of fixed MLC2a-/- ; Tg(ε-globin-eGFP) embryos show that vascular remodeling is impaired (Figure 4). MLC2a-/-; Tg(ε-globin-eGFP) are indistinguishable from wildtype or heterozygous littermates at 8.5 dpc; However, by 9.5 dpc MLC2a-/- null embryos still have an unremodeled capillary plexus while MLC2a +/+ and +/- embryos show a patterned vasculature with a hierarchical arrangement of large and small caliber vessels.

Although 8.5 dpc embryos appeared phenotypically indistinguishable from the wild type littermates, we hypothesized that the deficient yolk sac remodeling observed at 9.5 dpc was due to altered flow patterns occurring at 8.5 dpc. Thus, we characterized circulation in 8.5 dpc MLC2a$^{-/-}$ embryos. First, to determine if the onset of circulation is delayed in MLC2a$^{-/-}$ embryos compared to heterozygous or wildtype littermates, we examined embryos dissected at various somite stages to assess capillary plexus morphology and maturation (Table I). By the 10 somite stage, erythroblasts are routinely found throughout the plexus in wild-type and heterozygous embryos but erythroblasts were not seen consistently throughout the whole plexus until 12-13 somites in the MLC2a$^{-/-}$ embryos. Thus, the onset of erythroblast circulation appeared to be slightly delayed in MLC2a$^{-/-}$ embryos.

Next, we used time-lapse confocal microscopy to directly observe blood cell movement in MLC2a$^{-/-}$ embryos. Our time lapse data from wild-type embryos has shown that erythroblasts enter circulation in an intermittent fashion, with the majority of cells flowing with a net forward motion (Figure 1, Movie 1). Blood flow in the late 8.5 dpc yolk sac is typically pulsatile and fits a laminar profile (Jones et al., 2004). In contrast, time lapse sequences from similarly staged MLC2a$^{-/-}$ embryos showed that forward flow of erythroblasts was impaired. Erythroblasts were either stationary (data not shown) or moving in an oscillatory pattern, where there was an equal amount of retrograde and anterograde motion (Figure 5, Movie 2). Oscillatory motions of erythroblasts could easily be tracked from frame to frame using time lapse data acquired at 2 frames/sec (Figure 5B) because blood cells only move 3-5 cell diameters every 500ms, whereas the velocity of flowing erythroblasts in wild type yolk sacs must be measured using high

speed methods and ranges from 0.5 to 6 mm/s at this stage (Jones et al., 2004). Thus, instead of the normal fast, laminar flow pattern seen in wild type embryos, slow, oscillatory flow was observed in MLC2a$^{-/-}$ embryos.

To determine if the deviation from normal flow patterns were present prior to erythroblast entry into the circulation, we examined plasma flow in MLC2a$^{-/-}$ embryos using the FRAP technique. The measured perfusion coefficients are comparable between wild-type (Figure 3) and knock-out littermates at the 3 and 4 somite stages (data not shown), but by 6-9 somites, MLC2a$^{-/-}$ embryos show reduced plasma flow as compared to wild type littermates (Figure 6). By this stage, we have measured perfusion coefficients as high as 3835 $\mu m^2/s$ in wild type embryos (n=23) and often encounter regions with flow too fast to measure, whereas in knock-out embryos, all measured perfusion coefficients were nearly four times lower (below 1045 $\mu m^2/s$; n=28) and no vessels exhibited flow outside the measurable range. Of note, the perfusion coefficients measured in MLC2a$^{-/-}$ embryos are an order of magnitude larger than pure diffusion (51 $\mu m^2/s \pm 21$), indicating that plasma flow is present, but weak. These data show that poor cardiac function can be detected in very early mutant embryos, soon after the heart begins to beat and before any other phenotype is present.

Vascular remodeling requires fluid derived forces

Our high-resolution analysis of circulation in wildtype and mutant embryos revealed that there are two distinct types of flow during early cardiovascular development, a period of plasma flow, followed by the entry of erythroblasts into circulation, resulting in whole blood flow. In MLC2a-deficient embryos, both of these phases show reduced velocity, but from these data we cannot discern which function afforded by circulation is critical for vascular remodeling. Reduced plasma flow could limit the circulation of soluble factors and nutrients in embryonic and extraembryonic tissues. If soluble factors were sufficient to induce remodeling, restoring normal plasma flow alone should rescue remodeling. Alternatively, both oxygen transport and mechanical force depend on the hematocrit and if these are essential elements of the circulation than normal plasma

flow alone would not be sufficient to stimulate remodeling and the circulation of blood cells would be needed. Poor circulation of blood cells could increase hypoxia which could damage endothelial cells and inhibit remodeling. Alternatively, blood cell circulation could provide a necessary mechanical stimulus. Suspended particles in blood flow, such as erythroblasts, increase the overall effective viscosity of the blood (Chien et al., 1966) and mechanical forces such as shear stress depend on the apparent viscosity of the fluid. Therefore, we tested the role that erythroblasts play in vascular remodeling by experimentally sequestering blood cells within the blood islands during development. By reducing the hematocrit and the effective viscosity of the blood, without affecting plasma flow velocities we could determine if soluble factors in the plasma were sufficient for remodeling. To do this, we used focal applications of a polymerizing gel to immobilize the cells within the blood islands prior to the initiation of erythroblast circulation. Experiments were performed using Tg(ε-globin-eGFP) embryos to visualize the blood islands and monitor the extent of erythroblast circulation (Figure 7 C,F,I). Treated embryos were allowed to develop for 24h in roller culture and were subsequently assessed for vascular morphology, expansion of the yolk sac and the incidence of embryonic turning (see materials and methods). Controls were performed to ensure that each of the components of the polymer solutions were not toxic to embryos (Table 2).

In control and untreated embryos, a hierarchical vascular branching pattern with enlarged avascular spaces was observed in yolk sacs after 24h in culture (Figure 7, Table 2). Erythroblasts were robust in number and uniformly distributed within the vascular space (Figure 7C). When erythroblasts were prevented from leaving the blood islands, vascular remodeling did not take place (Table 2; Figure 7D,E,F). While control embryos formed the characteristic large and small caliber branched vessels, embryos with lowered hematocrit did not form any large vessels, only an immature plexus was observed in these embryos (Table 2;Figure 7E). In addition, embryos receiving this treatment grew and the yolk sac expanded, but the embryos did not turn (Table 2; Figure 7D,E,F). Occasionally, it appeared as if the initial steps in remodeling had occurred, characterized by the presence of elongated vascular tubes, but large diameter

vessels were never evident (Table 2). We attribute the slight variability in the vessel phenotype to variations in the extent of the gel treatment. Although most erythroblasts were immobilized, a small number of erythroblasts were often found in circulation following the treatment (see 7F). Gel treatment of the blood islands did not affect plasma flow in treated embryos. Plasma flow rates were comparable to normal, untreated embryos (Figure 8), indicating that the velocity of blood flow is not reduced. Thus, we conclude that the circulation of plasma alone is insufficient to drive vascular remodeling, arguing against the idea that vascular remodeling is triggered by a soluble factor carried by blood flow.

The above experiments show that the flow of erythroblasts is necessary for remodeling, but it is not clear whether remodeling requires the presence of blood cells for proper oxygenation or because of the mechanical force that is imparted by viscous flow. High molecular weight, synthetic sugars derived from plants, such as hetastarch, have been shown to increase the viscosity of solutions (Akers and Haidekker, 2004; Haidekker et al., 2002). Therefore, we injected a hetastarch solution into the circulation of embryos with sequestered blood cells to determine if raising the viscosity of the plasma could rescue the remodeling phenotype caused by the absence of circulating erythroblasts. We found that embryos treated with polymerizing gel and injected with hetastarch had clearly visible large, branched yolk sac vessels (Figure 7G,H,I), and were indistinguishable from wild type in terms of the size of the yolk sac (Table 2). Even embryo turning was restored in most cases when the viscosity of the blood was increased (Table 2). Thus, increasing the viscosity was sufficient to rescue the remodeling deficiency. These data show that mechanical force provides a signal responsible for triggering vascular remodeling in the yolk sac. Vascular remodeling can occur in the absence of blood cell circulation if the viscosity of the blood is adjusted to account for the drop in hematocrit showing that oxygen transport from the blood cells is not necessary for remodeling to take place. These data show that mechanical force is both necessary and sufficient to trigger vascular remodeling in the yolk sac of early mouse embryos.

Discussion

Physical forces imparted by blood flow are necessary and sufficient to induce vascular remodeling in the yolk sac

The main goal of these studies was to investigate the role of fluid-derived forces in vessel remodeling in an in vivo model system, the mouse embryo yolk sac. First, we characterized the onset of plasma and erythroblast flow to reveal the sequence of early events at the onset of circulation. Next, we examined plasma and erythroblast flow in mutants with impaired heart contractility to determine the flow patterns associated with poor yolk sac vascular remodeling. These two sets of investigations led us to formulate the hypothesis that the increase in apparent viscosity that occurs when blood cells enter the circulation results in a physical stimulus that triggers remodeling. We tested this hypothesis by first preventing blood cells from entering circulation. This treatment lowers the hematocrit and reduces the apparent viscosity of the blood. We found that sequestering the blood cells prevented vascular remodeling and embryo turning. These data showed that circulating blood cells were necessary for vascular remodeling, but we could not conclude that this effect was due to an alteration of the mechanical properties of the blood. Therefore, another set of experiments were performed in which blood cells were prevented from entering circulation but were replaced by injecting a plant starch to increase the viscosity of the plasma. This treatment rescued vascular remodeling and turning; therefore, changes in the viscosity of the blood, and hence the physical force exerted by the blood, is sufficient to induce vascular remodeling and embryonic turning in early mouse embryos. Since prolonged plasma flow was not sufficient to induce remodeling and remodeling could be triggered in the absence of oxygen carrying blood cells, we can firmly conclude that mechanical force, rather than soluble factors or changes in oxygen supply, provides the critical signal for vascular remodeling in the early mouse embryo.

Our work clearly shows that the mechanical force exerted by blood flow imparts an important signal for triggering vascular remodeling, although precisely which forces are

most important for vascular remodeling remain an open question since both shear stress and circumferential strain could be instructive. Shear stress, the frictional force exerted by blood flow is directly dependant on the apparent viscosity of the fluid and is likely to be a critical force acting on early endothelial cells. Shear stress has been shown to induce changes in gene expression, cytoskeletal architecture and cellular dynamics, including proliferation, in cultured endothelial cells (Garcia-Cardena et al., 2001; Helmke, 2005; Li et al., 2005; Orr et al., 2006; Resnick et al., 2002). Our previous research has shown that shear stress can be as high as 6 dyn/cm^2 in the mouse yolk sac during the first day of erythroblast circulation, within the range known to induce changes in cell morphology and upregulate shear stress responsive genes (Jones et al., 2004). Others have shown that non-erythroid, murine yolk sac cells extend cellular projections in the absence of flow but cease in the presence of low shear stress (1 dyn/cm^2) (Blatnik et al., 2005) and May et al (2004) have shown that endothelial cells in Titan mice, which have deficiencies in cardiac contraction, are not aligned along the vessel wall, suggesting a lack of sufficient shear stress. Despite the evidence supporting a role for shear stress, we cannot clearly state that shear stress is the only essential force at work in this system since circumferential strain may also play an important role in early development. Many experiments show that both types of force can induce similar physical and molecular changes in cultured endothelial cells (Lehoux and Tedgui, 1998; Kamiya and Togawa, 1980; Lie et al 2005; Orr et al 2006) and presently, we cannot distinguish a separate role for the two different types of force.

The initiation of murine blood circulation

Our analysis of flow in the early yolk sac indicates that erythroblast circulation is preceded by several hours of plasma flow, beginning as soon as the heart begins to beat. While some sources have described a patent heart tube that is present by 6 somites (See Ji et al 2003 and references therein), our experiments show that there is a functional connection between the heart and yolk sac vessels that is capable of supporting plasma flow by the time the heart begins to beat at 2-3 somites. Initially

plasma flow is slow, but significantly faster than diffusion, and increases in strength during early somite stages.

During the initial stages of circulation when blood consists of plasma alone, we suspect that endothelial cells are exposed to very low shear stress; however, we have not yet been able to measure shear stress exerted by plasma flow. Although these forces are insufficient to initiate remodeling, low levels of shear stress may influence early endothelial cells in other ways. Interestingly, recent evidence indicates that shear stress as low as 1.5 dyn/cm^2 can stimulate the differentiation of Flk-1+ cells in ES cell culture assays (Yamamoto et al., 2005) and can reduce the motility of yolk sac cells (Blatnik et al., 2005). Thus, it is possible that the initial period of plasma flow helps to facilitate the formation of endothelial cells or reduce the motility of endothelial cells so that they can form stable vessel networks. Further experiments will be needed to determine if plasma flow alone plays a specific in vivo role.

Plasma flow steadily increases during early somite stages and a small number of blood cells enter into circulation beginning at the 5-6 somite stage. We believe that blood cells begin to enter the circulation once sufficient force is generated by the flow to initiate blood cell movement and keep cells suspended. It is not clear whether the increase in force provided by stronger plasma flow is the only significant factor to influence the initial movement of blood cells. It is also possible that changes in cell adhesion between erythroblasts and surrounding cells facilitates erythroblast circulation, although this has not been investigated directly here.

Previous studies have investigated the onset of circulation in mouse embryos by examining the location of erythroblasts in fixed embryos (Ji et al., 2003; McGrath et al., 2003) and by assessing blood flow in the heart and dorsal aorta using high-resolution ultrasound (Ji et al., 2003; Phoon et al., 2000; Phoon et al., 2002). We have extended these studies by examining the movement of GFP-labeled erythroblasts directly in developing embryos using time-lapse confocal microscopy. We show that when primitive blood cells begin to move through the capillary plexus, they are not

consistently suspended in the flow and can come to rest, even adhering to cells that line early vessels. Interactions between individual blood cells and the walls of the microvessels in the plexus can increase the apparent viscosity by increasing the resistance to flow (see (Fung, 1997), Chapter 1.8, p13). Thus, it is likely that the forces exerted on individual endothelial cells may be quite diverse in different microenvironments within the plexus due to the particulate nature of the blood and the interactions between blood and endothelial cells. Given the potential for these types of interactions, it is interesting that vascular remodeling can be induced by changing the viscosity using a non-particulate suspension. These data indicate that the specific interactions between blood and endothelial cells may not be as important for vascular remodeling as the overall change in viscosity that occurs when the hematocrit increases.

Impaired circulation in MLC2a mutant embryos

Hemodynamic deficiencies in MLC2a mutant mice were quantitatively assessed using a combination of fluorescent transgenic marker strains and time-lapse confocal microscopy. By examining GFP+ erythroblasts in MLC2a null embryos, we observed that erythroblasts fill the yolk sac plexus, but at a slightly later stage than in wild type embryos. Without the benefit of time-lapse analysis, one might conclude that there was only a minor flow abnormality in these embryos, but time-lapse data of 11 somite MLC2a-/- embryos revealed that severe flow abnormalities were present. Instead of flowing in a pulsatile, forward pattern, erythroblasts in mutants oscillated back and forth with little net forward motion. We concluded that the slow oscillatory flow is sufficient to distribute blood cells from the blood islands into more proximal regions of the plexus, but insufficient to induce remodeling. Thus, time-lapse analysis was critical in understanding the nature of the flow deficiency present in mutant embryos.

Since blocking the flow of erythroblasts in wild type embryos phenocopies the remodeling defect seen in MLC2a mutant embryos, it is likely that remodeling fails because erythroblasts do not establish a normal laminar flow pattern, reducing the force

exerted by the flow. Alternatively, it is possible that the change in the flow pattern from laminar to oscillatory could also play a significant role. Culturing mouse embryos in KB-R7943, an NCX1 inhibitor, reduces cardiac function and leads to impaired yolk sac plexus remodeling (unpublished data). Blood cells do not oscillate back and forth, but do exhibit severely reduced motion suggesting that the reduced motion of erythroblasts, regardless of the pattern of flow, is to blame for the remodeling phenotype. It is not known whether the oscillations in MLC2a embryos have any effect on local endothelial cells. Cell proliferation, which is thought to be enhanced by oscillatory flow patterns (Chiu et al., 1998; Davies et al., 1986; Levesque et al., 1990), is similar between MLC2a and wildtype embryos (data not shown).

The oscillatory flow seen in MLC2a mutant embryos can be attributed to altered cardiac contraction. Although MLC2a RNA is expressed throughout the heart tube at 7.5dpc (Kubalak et al., 1994), MLC2a protein is only incorporated into atrial myofibrils (Chen et al., 1998). We believe that the oscillatory motion is due to the mismatch between the functional ventricle and the impaired atrium. In the absence of atrial contraction, net formward motion of the blood cannot be maintained. Thus, even though a clear morphological distinction between compartments is not evident in the mutant embryos at this stage (Huang et al., 2003), functional differences between the compartments can be detected by observing the blood flow pattern. Also, since poor plasma circulation is seen as early as 6-9 somites, it is clear that atrial contraction plays an important role in blood circulation very soon after the heart begins to beat.

Blood flow and embryo turning

Our analysis of hemodynamics in early mouse embryos has led to the interesting conclusion that proper blood flow is required for embryo turning. Although it is not entirely clear why embryos do not turn when circulation is impaired, we suspect that poor or absent circulation changes the tension within the yolk sac as it expands which could impair turning. Similar effects on turning were observed in MLC2a-deficient embryos (data not shown), however we are not aware whether this is a common feature

of mice with hemodynamic deficiencies since it generally not reported. The yolk sac of embryos treated to sequester the blood cells are slightly smaller than control embryos, indicating that the yolk sac has not expanded completely. In our initial analysis of optimal culture conditions for growing embryos in culture, we also noticed that turning was impaired when the yolk sac did not expand well (Jones et al 2002; unpublished observations). Alternatively, the lack of blood flow in the embryo proper may also be responsible for poor turning. At present little is known about how the mechanical forces needed for turning are generated within the embryo and surrounding tissues. Further investigations are needed to better understand the role of circulation.

The mechanical role of erythroblasts has been elusive

The role of primitive erythroblasts in early embryonic development has been difficult to ascertain. Mutations in several genes alter the development of blood cells in the early embryo, but other cell types are also affected. For instance, null mutations in E T-mod alters the mechanical properties of primitive erythroblasts; however, E T-mod embryos also have a defect in cardiac contractility that impairs circulation (Chu et al., 2003), so it is not known if altering blood cell structure could impair remodeling. Our data implies that anything that would affect the rheology of the blood cells would also cause remodeling to be abnormal. Also, many mutations that impair hematopoiesis in 8.5dpc embryos also alter angioblast differentiation and/or function (Oike et al., 1999; Oshima et al., 1996; Robb et al., 1995; Shalaby et al., 1995; Shivdasani et al., 1995; Suri et al., 1996). For instance, in Tal1/SCL mutants, hematopoeisis is severely impaired as embryos lack primitive erythroblasts and have defects in vascular remodeling (Robb et al., 1995; Shivdasani et al., 1995); however transgenic rescue and chimera experiments show Tal1/SCL is essential in endothelial cells for proper vascular remodeling (Visvader et al., 1998). It is suspected that severe anemia alone would also be sufficient to induce the same phenotype, but these conditions have never been encountered in genetically engineered embryos at such early stages.

The genetic basis for vascular remodeling

Recently, there have been great strides in identifying the molecular components of a shear stress transduction pathway, implicating many molecules that are expressed during vasculogenesis and vessel remodeling. Shear stress is thought to be sensed by PECAM-1, VE-Cadherin and VEGFR2 (Flk1) (Tzima et al., 2005) at the membrane which leads to the activation of c-Src (Jalali et al., 1998; Okuda et al., 1999) and the conversion of Integrins to high affinity forms (Tzima et al., 2002; Tzima et al., 2001; Tzima et al., 2003). Furthermore, Kruppel-like factor-2 (Klf2) has been shown to be upregulated in response to shear stress and a necessary transcription factor for the expression of flow regulated genes (Dekker et al., 2006; Dekker et al., 2005; Parmar et al., 2006). However, due to redundancies and earlier functions, mutant analysis has gleaned only limited information about the mechanisms of vessel remodeling. Surprisingly, PECAM-1 knock out mice are viable to adulthood (Duncan et al., 1999) suggesting that there may be functional redundancy in this pathway in vivo. VEGFR2 plays an earlier role in the differentiation of blood and endothelial cells (Shalaby et al., 1997; Shalaby et al., 1995) making it difficult to understand how it is involved in shear stress signaling, and Klf2-deficient embryos undergo vessel remodeling, but later in gestation have defects in vessel stability (Kuo et al 1997). Interestingly, VE-Cadherin-deficient embryos fail to remodel the initial vascular plexus (Carmeliet et al., 1999; Gory-Faure et al., 1999), which would be predicted based on the data shown here if endothelial cells could not properly sense shear stress. Instead of undergoing remodeling, endothelial cells become detached from each other and the basement membrane, and undergo apoptosis (Carmeliet et al., 1999). Although these data are consistent with a role in transducing mechanical signals in vivo, it is also possible that vascular remodeling fails because many endothelial cells die (Carmeliet et al., 1999). Further investigation is needed to determine if there is more direct evidence to implicate VE-Cadherin in shear stress-induction, such as whether shear stress-induced genes are abnormally regulated in yolk sac cells.

Conclusion

In this study, we have used novel methods to define a role for fluid derived mechanical signaling during embryonic development. By generating methods to manipulate the

physical properties of the early blood, we were able to show that the material properties of the blood may be more important than the metabolic function that blood plays later in development. Strategies such as these show the strength of combining both genetic and physiological methods to understand complex cardiovascular phenotypes and open the door to further investigation of shear stress signaling in vivo.

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Tables

Table 1 – Expansion of Blood Islands and Erythroblast Circulation: Comparison between wild type and MLC2a$^{-/-}$ Embryos.

	Somite stage	4	5	6	7	8	9	10	11	12	13	14-15
+/+ & +/-	Blood Islands Only	8	11	10	4							
	Expansion proximal to blood islands	4	11	15	19	14	13	4				
	Full plexus			2	5	10	12	14	12	11	12	17
	n=	12	22	27	28	24	25	18	12	11	12	17

	Somite stage	4	5	6	7	8	9	10	11	12	13	14-15
-/-	Blood Islands Only	6	4	2	5	1		1				
	Expansion proximal to blood islands	1		4	2	6	4	3	4			1
	Full plexus						2				5	2
	n=	7	4	6	7	7	6	4	4		5	3

Table 2 – Quantitative Analysis of Growth, Embryo Turning and Yolk Sac Vessel Remodeling in Treated Embryos.

Treatment	n	Area (mm^2)	Turn Score	Remodel Score
Control	23	5.23 ± 0.19	3.78 ± 0.32	4.13 ± 0.30
Low Hematocrit.	41	4.06 ± 0.12*	1.70 ± 0.15*	1.76 ± 0.16*
Low Hematocrit + Hetastarch	19	5.19 ± 0.28	2.68 ± 0.32	3.58 ± 0.30
Acrylamide only	5	4.93 ± 0.41	3.80 ± 0.49	4.20 ± 0.49
TEMED only	14	5.93 ± 0.28	4.00 ± 0.36	4.64 ± 0.20
Hetastarch only	12	6.05 ± 0.24	3.50 ± 0.44	4.25 ± 0.37

Figure Legend

Figure 1 – Initiation of Erythroblast Circulation. The initiation of erythroblast circulation was followed using time-lapse microscopy (Movie2) in a 6-somite mouse embryo expressing erythroblast specific GFP. The yolk sac (YS), and the heart (H) and somites (S) can be seen. Erythroblasts enter circulation within 11 frames. Some newly circulating erythroblasts exit the flow stream and remain stationary for various periods of time (arrows). Images were taken every 6 minutes at 10x magnification on a Zeiss LSM5 PASCAL for a total of 12.1 hrs.

Figure 2 – Initiation of Plasma Flow. 10,000MW fluorescent dextran was injected into the heart (Hrt) of early mouse embryos. The embryos were incubated for 10 minutes and images were taken using a Zeiss LSM5 PASCAL microscope at 5x magnification. The presence of fluorescence in the yolk sac after such a short incubation was interpreted as the result of flowing blood plasma. In most 2-somite embryos (5 out of 6 injected embryos), dextran remains localized to the heart (A and B; arrow in B). In one observed case, however, fluorescent dextran could be observed in the proximal yolk sac (ys) (C and D; arrow in D). Plasma circulation is consistently observed at the 3 somite stage onward (data not shown). A, C are images taken with brightfield illumination overlaid with fluorescence images of fluorescent-labeled dextran within the vessels (fluorescent microangiographs), B,D are the microangiographs only.

Figure 3 – Perfusion Coefficients of Wild-Type Embryos Measured using FRAP. Plasma flow magnitude in the early capillary network was determined between somite stages 3 and 8 by calculating a perfusion coefficient using Fluorescence Recovery After Photobleaching (FRAP). The upper range of perfusion coefficients increases as cardiogenesis progresses and at the 6 somite stage and later, flows too fast to measure

are observed (asterisk and arrow). We observed a range of values even within a given embryo since flow was reduced at sites farther from the heart.

Figure 4 – Phenotype of MLC2a$^{-/-}$ Embryos. Heterozygous (A, C, E) and knock-out (B, D, F) littermates at the 7 somite (A-B), 10 somite (C-D) and approximately 23 somite stage (9.5 dpc) (E-F). The capillary plexus is demarcated via GFP-expressing erythroblasts. In the knockout embryos, blood islands form (A,B) and erythroblasts enter the circulation (C,D), indicating that vascular development in MLC2a -/- embryos is not impaired through the 10 somite stage . However the plexus retains the immature phenotype and fails to remodel by embryonic day 9.5 in the mutant embryos (E,F) (F).

Figure 5 – Erythroblast Motion in MLC2a$^{-/-}$ embryos. The motion of erythroblasts (green) within vessels of MLC2a$^{-/-}$ embryos was imaged at 2Hz using a 40x objective lens on a Zeiss LSM5 PASCAL. Panels represent every 5th frame. The motion of individual erythroblasts were tracked and plotted (B), illustrating that erythroblasts oscillate with as much retrograde motion as anterograde.

Figure 6 – Perfusion Coefficients of Embryos with Impaired Cardiac Function. Fluorescence Recovery After Photobleaching (FRAP) was used to calculate perfusion coefficients within the early embryonic blood vessels in MLC2a$^{-/-}$ embryos and wild type embryos at the 6-9 somite stage. Perfusion coefficient ranges were significantly lower mutant embryos compared to wild type embryos. While some coefficients in wildtype embryos were too fast to measure, all flows could be measured in mutant embryos. The average measured flow rate was 1601$\pm$267 $\mu m^2/s$ for control embryos and 312 $\pm$ 47 $\mu m^2/s$ for MLC2a$^{-/-}$ embryos.

Figure 7 – Plasma Viscosity Alters Yolk Sac Remodeling. Embryos were harvested at 8.25 dpc, placed in roller culture for 24 hours, and injected with red fluorescent dextran to create a fluorescent microangiograph (B,E and H). Control embryos turned (A) and

yolk sac vessels remodeled into a hierarchical, branched phenotype (B). A substantial eGFP-positive erythroblast population was evident in all parts of the yolk sac vasculature (C). Embryos with erythroblasts sequestered in the blood islands often did not turn but continued to develop after 24 hours in culture conditions (D). The yolk sac vasculature did not remodel and retained features of an immature plexus (E). Erythroblasts remained confined to the site of the blood islands (F). Embryo turning was restored (G) and yolk sac remodeling was rescued (H) in embryos with sequestered erythroblasts (I) after injection of Hetastarch. Scale bar = 50µm.

Figure 8 – Perfusion Coefficients in Embryos Experiencing Low Viscosity. Fluorescence Recovery After Photobleaching (FRAP) was used to calculate perfusion coefficients for the plasma motion in wild-type embryos and embryos with erythroblasts sequestered in the blood islands. The perfusion coefficient ranges and mean values were similar in wild-type and polymerized embryos, indicating that plasma velocity is normal following the treatment to sequester the erythroblasts.

Figure 1

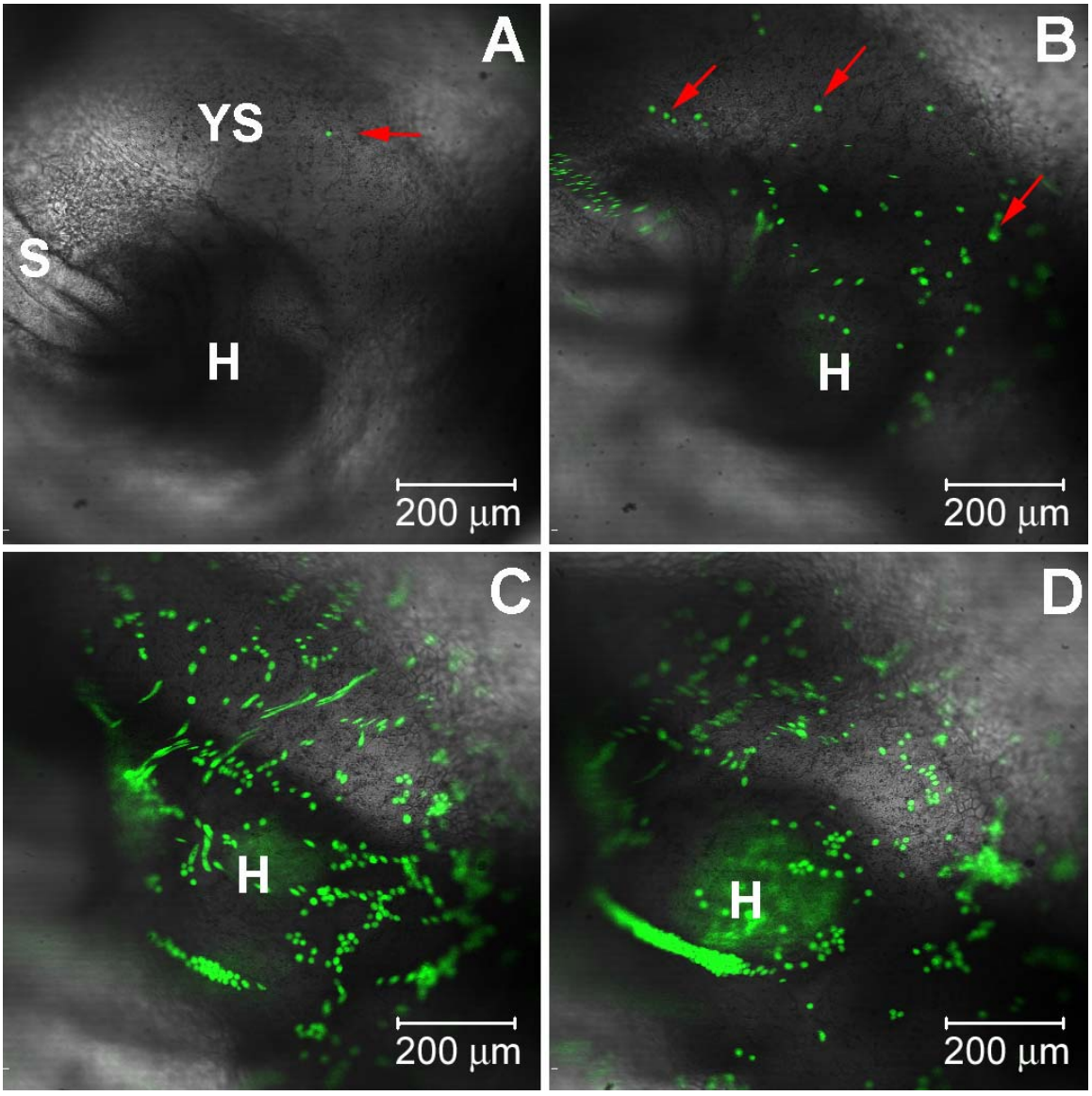
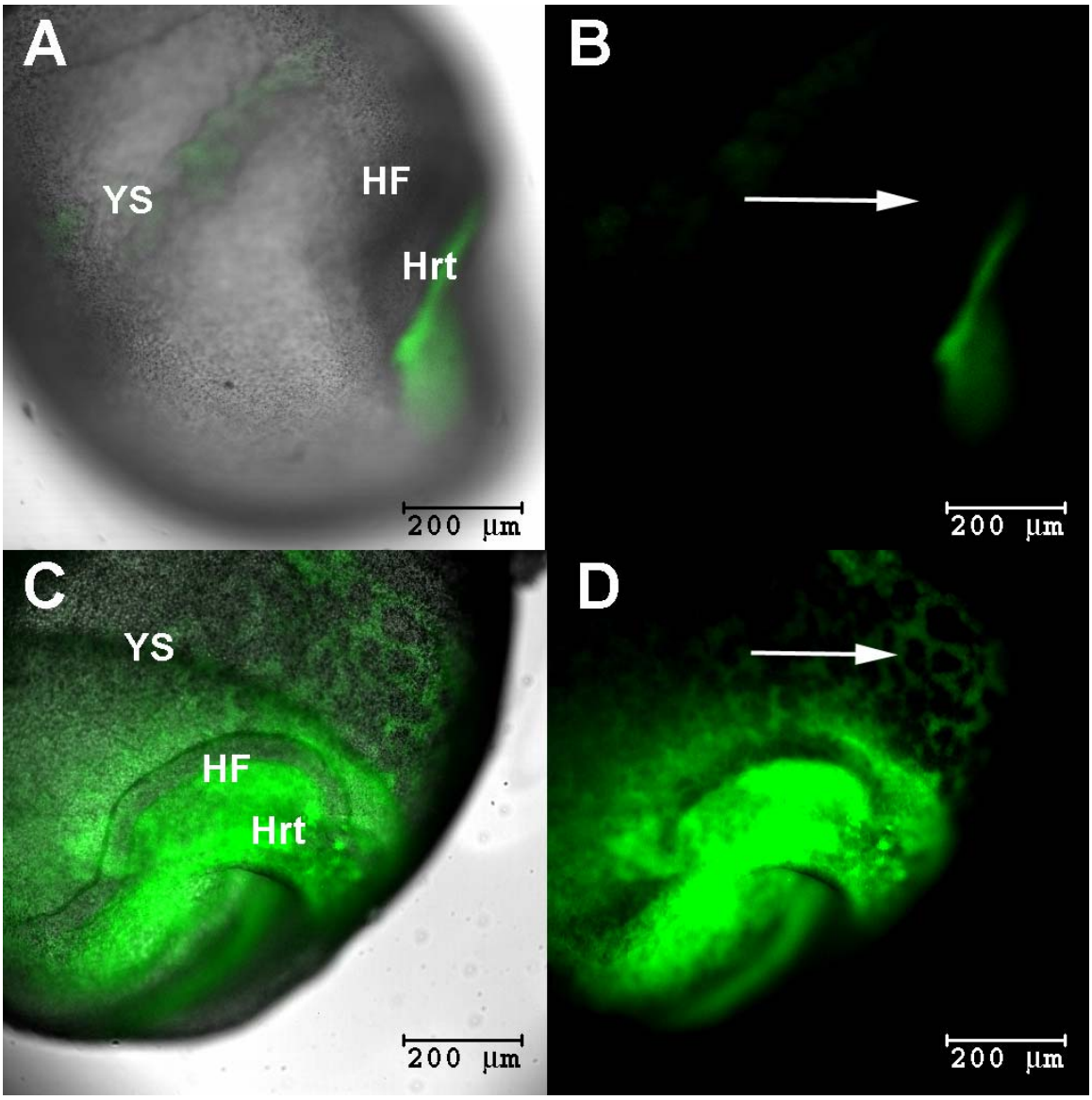


Figure 2



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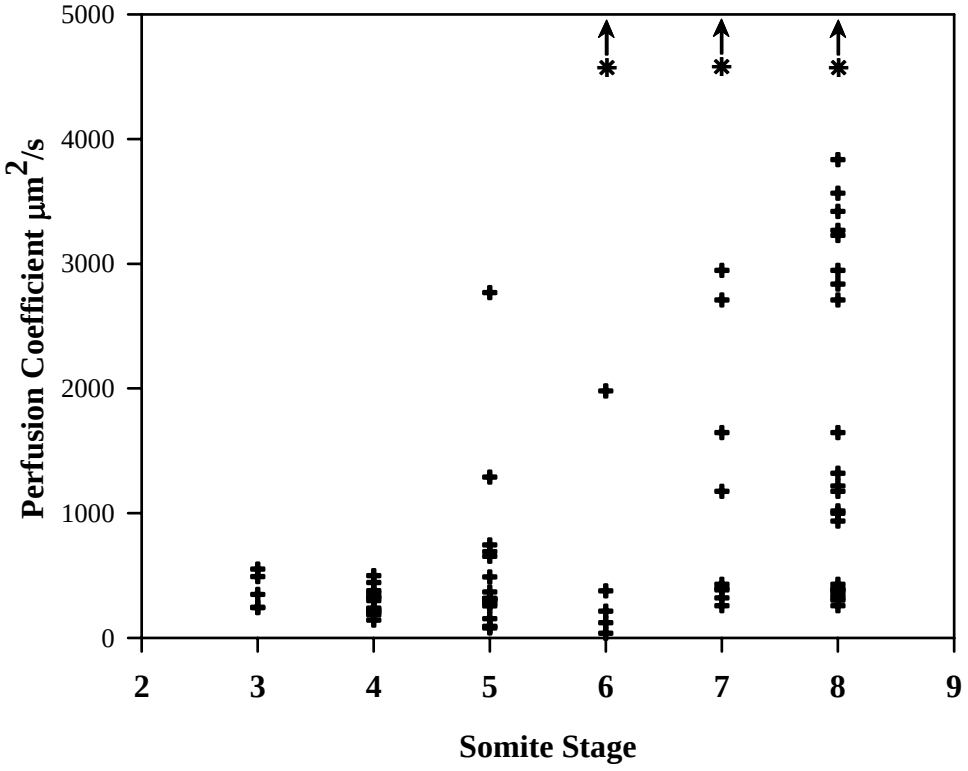

5000
4000
3000
2000
1000
0
Perfusion Coefficient μm2/s
2
3
4
5
6
7
8
9
Somite Stage

Figure 4

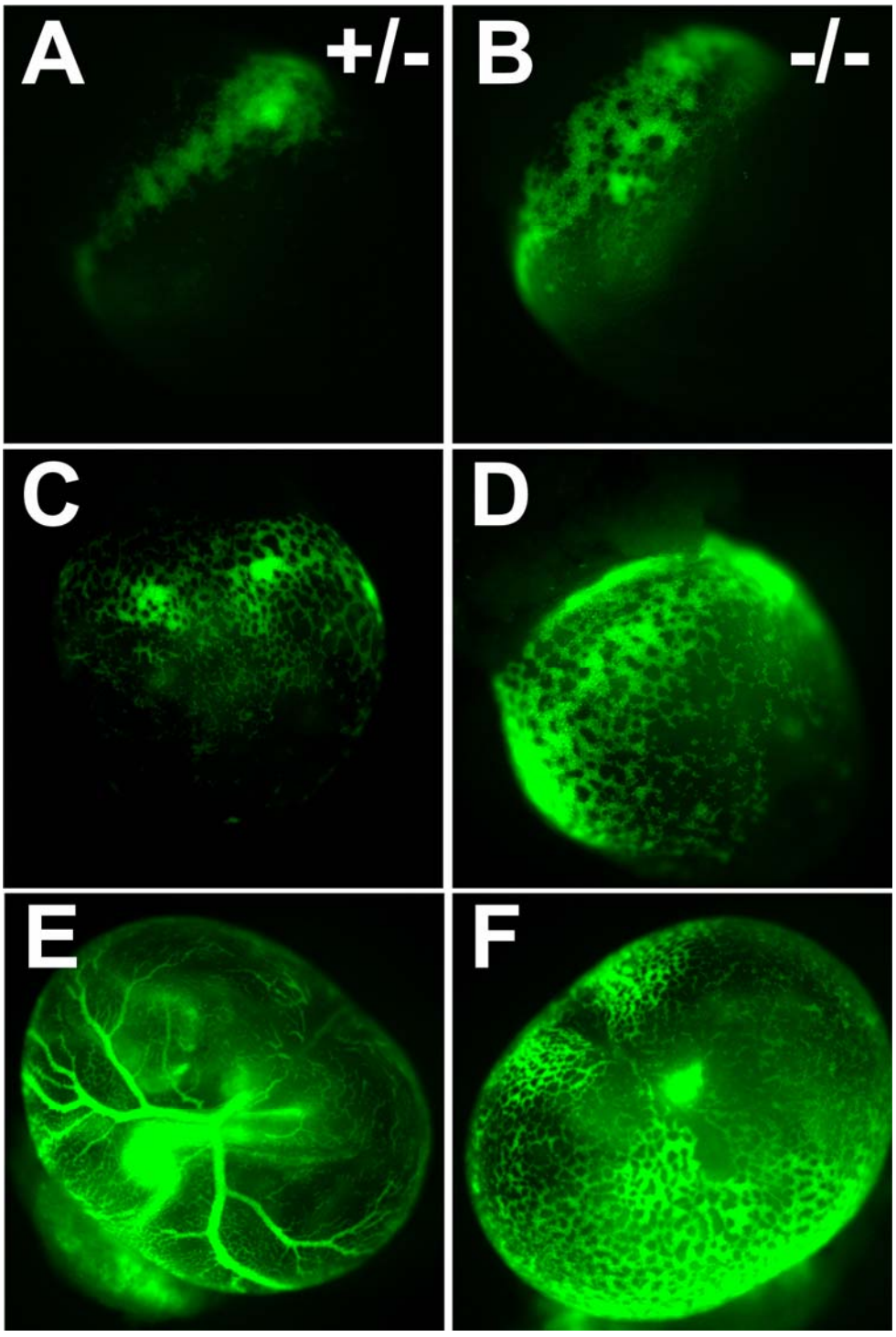


Figure 5

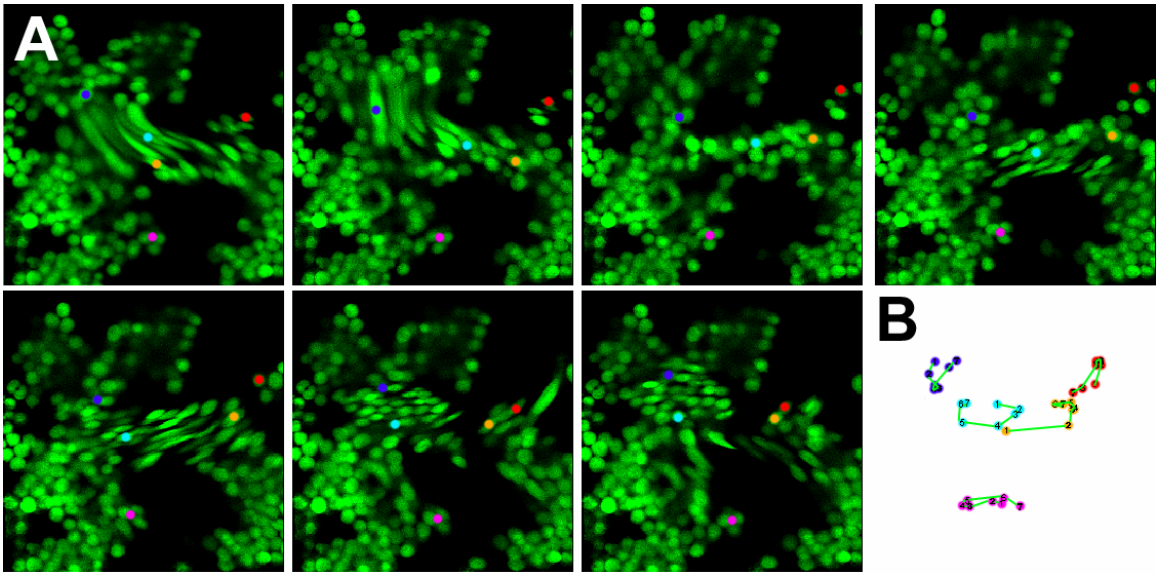
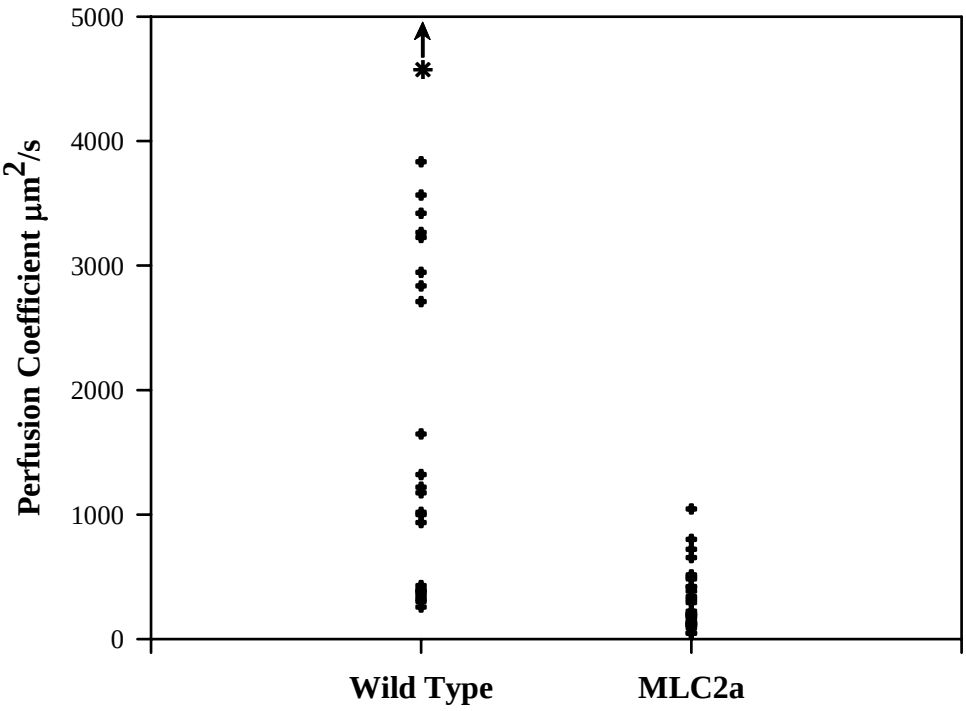


Figure 6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4000
3000
2000
1000
0
Perfusion Coefficient µm2/s
Wild Type
MLC2a

Figure 7

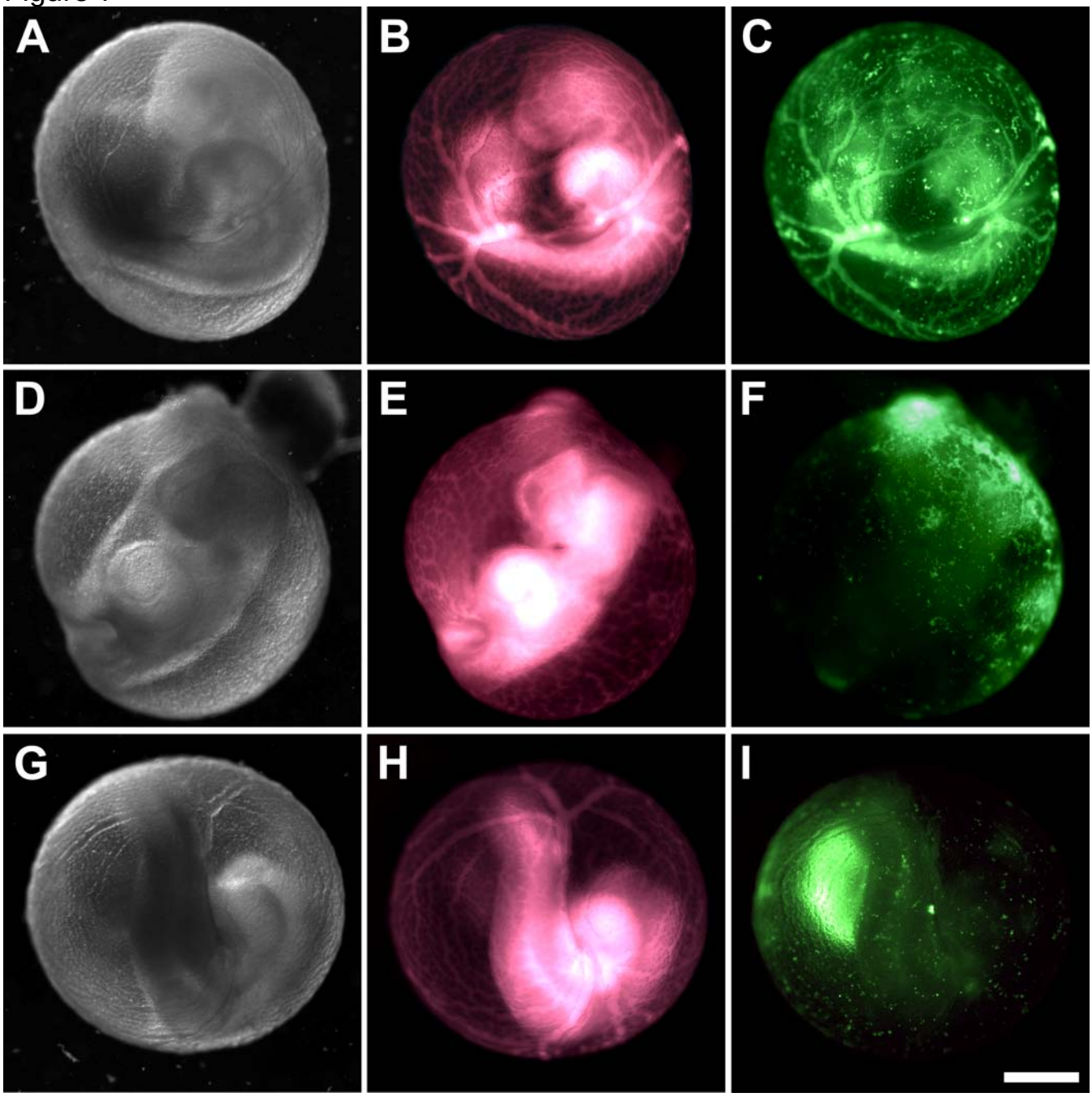
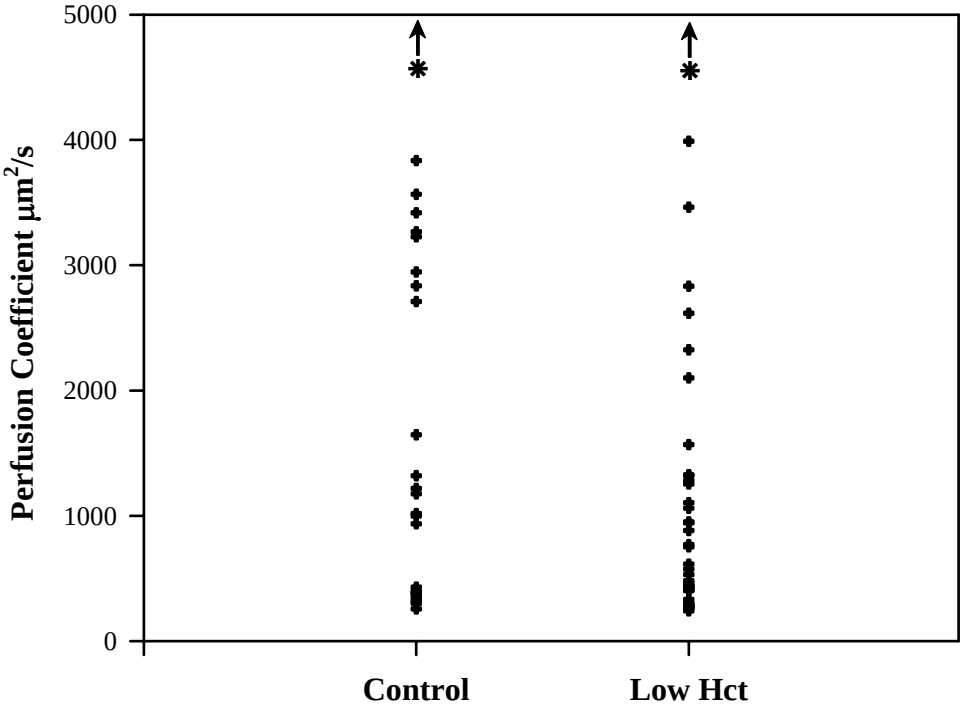


Figure 8


5000
4000
3000
2000
1000
0
Perfusion Coefficient μm²/s
Control
Low Hct